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The role of *Atonal* transcription factors in the development of mechanosensitive cells

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1: *Atonal* genes and mechanosensory cells

Two bHLH gene families are associated with sensory cells across metazoans – the *achaete-scute* and *atonal* families. Of these, the *atonal* genes are particularly strongly linked with the specification of photo- and mechanoreceptor cells [1]. The *atonal* gene was discovered in *Drosophila* as a proneural transcription factor for mechanoreceptive neurons and photoreceptor cells [2,3]. Jellyfish *atonal* homologues are expressed in photoreceptive and mechanosensory cells [4]. In vertebrates, these two key functions of *atonal* have been separated by gene duplication, such that *Atoh7* is required for retinal ganglion cells and *Atoh1* for mechanosensitive cells.

Invertebrates such as *Drosophila* use *atonal* to generate intrinsically mechanosensitive neurons called chordotonal (Ch) neurons. These bipolar neurons have a dendrite terminating in a sensory cilium that harbors the mechanoreceptive machinery (Fig. 1A). In some parts of the fly's body these are proprioceptive, while in the antenna they are auditory. In contrast, some vertebrate sensory systems have split the function of the mechanosensitive neuron into a sensory receptor cell (such as hair cells and Merkel cells) that makes synaptic connections with a sensory neuron that is no longer mechanosensitive (Fig. 1B). The vertebrate *Atoh1* genes are expressed in many mechanosensitive progenitor cells and are necessary for the development of hair cells of the inner ear and lateral line [5-7], and touch-sensitive Merkel cells in the skin [8,9]. Despite obvious structural differences, there are persuasive indications that Ch neurons and hair cells are derived from an ancestral *atonal*-specified mechanosensitive cell type [1,10,11].

A parallel argument is that *atonal* genes (*atonal/Atoh7*) are also anciently connected to photoreceptive cell development [12]. Indeed, it has been proposed that photoreceptive and mechanosensitive cells are linked even further back in an *atonal*-dependent proto-sensory organ [13]. To this could be added chemosensation, since *Drosophila atonal* and its in-paralogue *amos* are also required for olfactory neurons [14]. Similarly, the *C. elegans* homologue of *atonal*, *lin-32*, is required for pairs of mechanosensitive and chemosensitive neurons in the male tail [15]. Comparisons of the sensory transduction apparatus also hint at molecular

connections between these sensory modalities. For instance, Ch neurons express visual rhodopsins and olfactory ionotropic channels, both of which contribute to auditory sensory transduction [16].

The degree of conservation in the mechanosensory function of *atonal/Atoh1* is striking: *Drosophila atonal* can fully rescue *Atoh1* null mutant mice [17]; conversely, mouse *Atoh1* can partially rescue *atonal* mutant flies [8]. In this review, we focus on these mechanosensory roles.

2: *Drosophila Atonal* and the development of sensory neurons

Ch neurons form part of internal sense organs that mediate proprioception (Ch organs typically located to respond to joint or body movement), and hearing and gravitaxis (the large Ch neuron array of Johnston's Organ in the antenna) [18]. During the formation of the precursors of these neurons within the ectoderm (sense organ precursors, SOPs), *atonal* functions as a 'typical' proneural gene in that its expression is necessary and sufficient for SOP specification [2]. It is transiently expressed in ectodermal groups of cells (proneural clusters (PNCs)) and then is restricted to a subset – the SOPs) – by Notch-mediated by lateral inhibition. After commitment, each SOP divides several times asymmetrically to give the 4-6 cells of a unit Ch organ, 1-2 of which differentiation as Ch neurons. Failure to generate Ch SOPs in *atonal* mutants results in individuals that are deaf and exhibit uncoordinated locomotion [19-21].

2.1: The regulation of atonal *in mechanosensory cell development*

atonal transcription is spatially and temporally regulated in two distinct phases of expression. In the first phase, *atonal* is activated by combinations of regionally expressed patterning factors and signals to give the PNCs at locations corresponding to the ultimate positions of Ch organs. In the second phase, expression becomes restricted to the SOPs – it is upregulated in these cells and downregulated in the remaining cells of each PNC during lateral inhibition. This entails positive autoregulation in the SOPs and Notch inhibition in the remaining cells, initiated by the SOPs themselves. In addition to the inhibitory

signal, the SOPs also send out an EGFR pathway recruitment signal that causes upregulation of *atonal* in adjacent PNC cells [22-24]. The balance between Notch inhibition and EGFR induction determines the proportion of SOPs deriving from a PNC, and is at least part of the reason why Ch neurons can exist in large cohesive arrays, such as Johnston's Organ.

The *cis* regulation of *atonal* is achieved through extensive batteries of enhancer elements up- and downstream. In general, separate elements are required for activation of *atonal* in different locations, and also for the different temporal phases of expression [25]. In particular, it appears that the 3' region contains enhancer elements to initiate PNC expression whereas enhancers in the 5' region control upregulation in the SOPs. The 5' region contains separable autoregulatory enhancers for *atonal* upregulation in different regions (leg, eye, antenna, etc). Only one of these 5' enhancers has been characterised in any detail. This enhancer responds directly to the EGFR recruitment signal outlined above, in both the embryo and the leg imaginal disc. Within the enhancer are adjacent binding sites for Atonal and Pointed proteins (the ETS transcription factor activated by EGFR signaling) [26]. Thus this enhancer is activated in cells within the PNC (which are therefore expressing *atonal* at a low level) that receive EGFR signaling from previously selected SOPs. In other words, for this enhancer *atonal* autoregulation is contingent on EGFR signaling.

Another component of *atonal* autoregulation is the bifunctional Zn-finger protein, Senseless. This is a DNA-binding transcriptional repressor in the PNCs, but becomes a coactivator when bound to *atonal* or other proneural proteins [27,28]. *senseless* is also a target gene of *atonal*. Thus, *atonal* activates *senseless* expression, which then enhances *atonal* protein activity, thereby forming a positive feedback loop that helps to overcome Notch inhibition in SOPs.

Initial activation of *atonal* expression is directed by the 3' flanking region. The expectation was that this would contain different regulatory elements to initiate *atonal* expression in each of its different domains (antennal, leg, eye), but instead it appeared to comprise a single enhancer element that responds to the confluence of *dpp* (BMP) and *wg* (Wnt) signaling at each of these locations, as well a temporal ecdysone

signal to trigger expression at the appropriate time of development [13,25]. An intriguing explanation proposed for this was that all *atonal*-dependent sensory organs share a common developmental programme, and that cellular diversification arises through the later action of region-specific transcription factors such as *Pax* and *Hox* genes [13]. This developmental model contributed to the evolutionary idea of an *atonal*-dependent proto-sensory organ as discussed above. However, subsequent studies have shown that this characterisation of *atonal's* regulation is simplistic since the 3' region can be split into separate elements for different expression domains [29]. The Ch neuron elements have not be characterised, but one element for the eye has binding sites for *eyeless* (*Pax6*) and *sine oculis* (*Six* gene) proteins [29,30]. Therefore, region-specific 'patterning' factors are likely to initiate *atonal* activation in combination with spatial and temporal signals. In the adult and larval eyes (and possibly also Ch neurons), *hedgehog* signaling is also required for *atonal* regulation [31,32], but it is not yet known whether this regulation is direct. Intriguingly, the trithorax-group chromatin remodeling factors are required in the eye specifically for *atonal* expression [33].

2.2: How atonal *regulates target genes*

Like other proneural bHLH factors, *atonal* regulates known target genes as a transcriptional activator by binding to E box motifs as a heterodimer with an E protein, Daughterless [2]. Investigation of several known or suspected target genes suggested that *atonal* regulates targets via an extended variant of the generic E box (A A/T C A G/T G T G T/G) [34]. A more recent systematic computational identification of putative target genes (albeit focused on the eye) suggested a related target gene-associated motif of G/A A C A C/G C T G C/T. In each case, the motif differs from the E box associated with target genes of the *achaete-scute* family (binding site: G C A G C/G T G T/G), which goes part way to explaining how *atonal* and *achaete-scute* differ in function and why they cannot substitute for each other [35].

The function of *atonal* protein is clearly context-dependent (i.e. it specifies mechanoreceptors, olfactory receptors and photoreceptors in different locations) implying that some proportion of *atonal's* target genes

will differ according to developmental context. As yet, there is little direct evidence of how *atonal's* activity may be modulated by context, but we may expect that other region-specific transcription factors will be involved, either through binding to adjacent sites in *atonal*-dependent enhancers (combinatorial control), or through binding to and altering *atonal's* DNA binding specificity directly (specificity coactivators). Indeed, there is some evidence that different *atonal* binding sites can support quite distinct expression patterns in reporter gene studies [34].

2.3: The function of atonal *in mechanosensory neurons*

What can target genes tell us about *atonal's* function in mechanosensory cells? Clearly it regulates SOP specification but what about subsequent development and differentiation? Whilst several studies have sought to find target genes of proneural proteins, so far few have been directed specifically at *atonal*. The most comprehensive attempt to identify direct target genes has been the combined transcriptome and computational analysis of Hassan and colleagues [36]. Although based on *atonal's* function in the eye, most of the validated targets were expressed both in the eye and in Ch neurons, suggesting once more that the *atonal*-dependent gene expression programme has many common features in the different sensory organs.

Further functional genomic approaches have aimed not specifically at identifying direct *atonal* targets, but at the more general question of what are the pathways activated in mechanosensory differentiation. A recent study identified the *timecourse* of transcriptome changes downstream of *atonal* function in embryonic Ch cells [37]. GFP-tagged Ch cells were isolated from embryos at 1-hour intervals of development and subjected to microarray analysis. The most striking finding was that the predominant programme activated in Ch cells is associated with constructing the sensory cilium: at later timepoints, some 40% of the top 100 differentially expressed genes were associated with ciliogenesis or cilia function. Moreover, all *Drosophila* homologues of known ciliogenesis genes are upregulated in Ch neuron transcriptome. This leads to the question of how this pathway is regulated by *atonal*. The transcriptome

analysis suggested two key transcription factors are activated by *atonal*. One is Rfx, a winged helix factor that regulates many ciliogenic genes in a variety of organisms, including in *Drosophila* sensory neurons [38]. The second is Fd3F, a FOX transcription factor. It was subsequently found that Fd3F regulates genes for specialisation of the Ch neuron cilium, notably genes of the intraflagellar transport A (IFT-A) complex, which is required to delineate the distinct motile and non-motile zones of the cilium (Fig. 1A), as well as genes for axonemal dyneins and associated TRPV channels, which populate the motile zone [39]. The dyneins contribute to hearing as part of the sensory transduction machinery, most likely as adaptation motors mediating amplification of sound reception via ciliary oscillations [16]. Loss of these specializations in *fd3F* mutant flies results in dysfunctional Ch neurons with immotile cilia leading to deafness and uncoordination [39].

Thus, *atonal* activates two transcription factors that work together to switch on the genes for the construction a major aspect of the Ch neuron – its mechanosensory cilium. In addition to this regulatory relay, a proportion of ciliogenic genes were found to be expressed surprisingly soon after Ch cell specification, and it was shown that at least one of these differentiation genes is a direct target of *atonal* [37,40].

It is striking that clear links were made between *atonal* and the mechanosensory apparatus, thereby raising the question of whether *Atoh1* conserves such links in hair cells. Certainly, in the zebrafish otic vesicle, the Fd3F relative, *Foxj1b,* is necessary for the kinocilium [41], which is required for correct development of the microvilli-based stereocilia and for otolith tethering. Although it is itself immotile, the kinocilium is thought to be derived from a motile cilium, As yet there is no information on the regulation of *Foxj1* genes in the vertebrate ear, nor on the role of Rfx genes.

A further approach has identified genes expressed in the Ch organs of adult antennae (therefore, auditory genes) by comparing gene expression in wild-type and *atonal* mutants [16]. Some 274 auditory organ genes were identified. Of 42 genes analysed further, 27 showed defective auditory reception, including some

homologues associated with human deafness. As mentioned above, a most surprising finding was that the 'auditory genes' included olfactory ionotropic receptors and visual rhodopsins. It will be interesting to determine whether *atonal* triggers a common pathway to regulate these genes in the three *atonal*-dependent sensory cell types. Interestingly, visual signal transduction genes have been shown to be required for thermosensation [42], whilst larval Ch neurons have been demonstrated to be thermoreceptive, allowing larvae to discriminate between their favored temperature (17.5^{o}C) and slightly lower temperatures (down to 14^{o}C) [43]. If this extends to adults, there is the possibility of a link between TRPV channels and visual signal transduction apparatus.

Together, the developmental and adult mechanosensory gene data provide an excellent resource for exploring the regulatory network downstream of *atonal* and for providing clues to hair cell pathways downstream of *Atoh1*.

3: The role of vertebrate *Atoh1* genes in the development of mechanosensory hair cells

In this section, we focus on the factors that regulate *Atoh1* expression in hair cell progenitors and discuss how *Atoh1* regulates hair cell differentiation and survival through its known targets.

3.1: Do vertebrate Atoh1 homologues function as proneural genes?

The *Drosophila atonal* gene fulfills several criteria of a proneural gene – its expression precedes and coincides with the selection of sensory neuronal progenitors, its expression is regulated by Notch-mediated lateral inhibition and its function is both necessary and sufficient for the development of those progenitors [44]. The situation is more complicated in some vertebrates, as it is less clear if *Atoh1* genes mark committed hair cell progenitors as opposed to multipotent progenitor cells capable of forming hair cells, supporting cells or neurons [45]. This is an important question since it impinges on *Atoh1's* potential in hair cell replacement therapies (see later). We deal first with zebrafish, which has two *atonal* homologues,

atoh1a and *atoh1b. Atoh1b* is expressed broadly in the otic placode before the differentiation of neurons and hair cells and is rapidly refined to two patches presaging distinct sensory epithelia [46]. It is necessary for the development of tether cells, precocious hair cells that seed and localize the formation of otoliths [46,47], but not for the development of the majority of later hair cells in the ear, nor of lateral line neuromasts [46]. In contrast, *atoh1a* is expressed later in the progenitors of the majority of zebrafish hair cells and lateral line neuromasts [48,49], and is necessary for their development, but not the development of the precocious *atoh1b*-dependent tether cells [7,46]. As might be expected from this division of labor between two closely related *atonal* homologues, knockdown of both genes in zebrafish eliminates both early- and late-developing hair cells (Millimaki et al., 2007). Conversely, ectopic expression of *atoh1a*, either constitutively or transiently, causes expanded regions of hair cells in the zebrafish ear [46,50]. These results, together with the broad and early expression of *atoh1b* in the otic placode and the negative regulation of zebrafish *atoh1* genes by Notch signaling (Millimaki et al., 2007) suggest that fish *atoh1* homologues act as true proneural genes.

In mammals, *Atoh1* is clearly necessary for hair cell formation [5,51,52], and ectopic expression of *Atoh1* is sufficient to induce ectopic hair cells in at least some non-sensory parts of the inner ear [53-55]. However, it is less clear whether *Atoh1* is expressed only in committed hair cell progenitors or in progenitors capable of giving rise to several cell types. A number of methods have been used to identify the onset of *Atoh1* expression in the inner ear, including in situ hybridization for *Atoh1* mRNA [56], antibody staining of Atoh1 protein [51], GFP-expressing reporter mice driven by the *Atoh1* autoregulatory enhancer [57] and b-galactosidase expression driven from *Atoh1* knockout alleles [58]. These methods give somewhat contradictory results [59], with b-galactosidase driven from the *Atoh1* locus being expressed in a broad domain of sensory precursor cells, whereas Atoh1 protein or GFP expression from *Atoh1*-GFP mice suggesting that *Atoh1* is expressed in nascent hair cells.

A partial resolution of these data has come from recently developed transgenic mice in which either an *Atoh1-GFP* fusion gene or Cre recombinase were knocked into the *Atoh1* locus [60,61]. Crossing *Atoh1*-Cre

knock-in mice with Cre reporter lines show labeling of supporting cells [61]. Although this can be interpreted as *Atoh1* marking a bipotential hair cell-supporting cell progenitor, two cautionary points should be considered. First, the number of supporting cells labeled by recombination in the different sensory organs of the ear varied widely, from about 60 per 100 hair cells in the organ of Corti to only 7 per 100 hair cells in the utriclar macula. Second, the pattern of Cre-labeled supporting cells in each organ is essentially random, with no obvious anatomical position or supporting cell sub-type being preferentially labeled (Yang et al., 2010; L. Gan, personal communication). One interpretation of these data is that the mammalian *Atoh1* gene is expressed in hair cell progenitors immediately before they commit to a hair cell fate, but that some of these *Atoh1*-expressing cells can be re-directed to a supporting cell fate, most likely by Notch-mediated lateral inhibition. The labeling of supporting cells by *Atoh1*-Cre mice would therefore vary according to the speed and efficiency with which Notch-mediated fate decisions are made as hair cells and supporting cells differentiate. Since the arrangement of hair cells and supporting cells in the organ of Corti is far more precise than in other sensory organs, it is reasonable to suggest that the higher number of Cre-labeled supporting cells in the organ of Corti is a consequence of the greater fine-tuning of hair cell and supporting cell numbers that occurs during the development of this structure.

3.2: What are the signals that activate Atoh1?

Although Atoh1 occupies a pivotal role in hair cell differentiation, very little is known of the factors that directly regulate its expression in differentiating prosensory cells. Several well-characterized signaling pathways have been shown to modulate hair cell numbers in the inner ear, such as Wnt, BMP, FGF and Shh signals [62-69]. However, it is not clear how many of these signals regulate *Atoh1* transcription directly, post-translationally modify the Atoh1 protein, or regulate other factors that co-operate with or control Atoh1 [59]. Further insights into how *Atoh1* is regulated have been gleaned from study of its well-characterized autoregulatory enhancer located downstream of the coding region (Fig. 2) [70]. This enhancer has E-box and N-box binding sites [70], and it is known that Atoh1 can itself activate this enhancer through E-box binding, and that this autoregulation is necessary for the maintenance of *Atoh1* transcription in hair cells [56].

It is known that members of the Hes and Hey gene family, which are able to bind to N-box sequences, can repress hair cell differentiation [71,72] and negatively regulate *Atoh1* expression [73,74], but it is not yet clear whether this is due to direct binding of Hes or Hey factors to the *Atoh1* enhancer, or by other mechanisms, such as sequestration of Atoh1 binding partners. The *Atoh1* autoregulatory enhancer can bind transcription factors known to be downstream of several signaling pathways, such as Wnt (b-catenin and Cdx2; [75,76] and BMP (Zic1 and Cdx2; [76,77]. A number of other transcription factor binding sites have been identified in the *Atoh1* enhancer [75-80], in particular for Sox2 [81]. Sox2 can form a transcriptional complex with Six1 and Eya1 that is sufficient to activate *Atoh1* when electroporated into the greater epithelial ridge (GER) of the mouse cochlea [81]. *Sox2* can also act by itself to activate *Atoh1* expression in the otocyst of chicken embryos [82], and *Six1* and *Eya1* can also induce *Atoh1* in the absence of *Sox2*, albeit at lower efficiency [81]. Since *Sox2, Six1* and *Eya1* are all expressed more broadly in the prosensory domain of the cochlear duct prior to the differentiation of hair cells, it is likely that additional signals are required to allow this transcriptional complex to activate *Atoh1* expression specifically in nascent hair cells. Interestingly, it has recently been shown that *Sox2, Six1* and *Eya1* act together in a context-dependent fashion in the inner ear, as they are also able to direct differentiation of neurons in the GER in the presence of the SWI/SNF chromatin remodeling complex [83]. In this context, they activate transcription of *Neurog1*, a neuron-specific bHLH factor that is closely related to *Atoh1*, but not *Atoh1* itself.

The induction of *Atoh1* and the production of hair cells occur over an extended period of time in all inner ear sensory organs. In the vestibular system, differentiation begins in the center of each sensory patch, with extra hair cells being added peripherally over time. In the case of the mouse utricular macula, the first *Atoh1*-expressing cells can be observed at embryonic day 11, but the last hair cells are not added until 14 days after birth [56,84,85]. In the cochlea, *Atoh1* is expressed in a basal-apical gradient starting in the mid-basal region at E13.5 and terminating in the apex 5-6 days later [57]. This sequential propagation of differentiation can occur correctly even in pieces of cochlea that have been mechanically separated and maintained in organ culture [86]. The basal-apical gradient of differentiation can be perturbed in mice carrying mutations for either *NeuroD* or *Neurog1* [87,88], where precocious *Atoh1* expression and

differentiation of hair cells is seen as early as E14.5. Neither *Neurod1* nor *Neurog1* are expressed in the cochlea at detectable levels during this period of differentiation, suggesting they may regulate the timing of *Atoh1* expression indirectly. Since both mouse mutants lack most of the spiral ganglion, it is possible that this releases signals that regulate *Atoh1* induction. Interestingly, *Shh* is expressed transiently in the spiral ganglion, disappearing in a basal-apical sequence from E13.5 onwards [89]. This observation, together with the finding that inhibition of *Shh* signaling can increase hair cell production in the cochlea [62] suggests that *Shh* may be one of the factors that regulates the timing of *Atoh1* expression in the correct basal-apical sequence in the cochlea. Since *Shh* is required at earlier stages in ear development [90], conditional deletion of *Shh* will be required to test this hypothesis.

3.3: How does Atoh1 regulate hair cell differentiation and survival?

At present, we know very little about mechanisms by which *Atoh1* regulates hair cell differentiation. A recent study which identified direct targets of Atoh1 in cerebellar granule cells suggested that it not only regulates downstream transcription factors, but also genes involved in many other cellular processes, such as cell division, chromosomal organization, metabolism, cell migration and cell adhesion [91]. In the context of hair cells, it is possible that *Atoh1* may simply regulate generic aspects of hair cell differentiation (for example, regulating apical-basal polarity, components of the mechanotransduction apparatus or actin polymerization), and that other factors may co-operate with *Atoh1* or act in parallel to regulate genes specific to particular classes of hair cell – for example, inner versus outer cochlear hair cells, or type I versus type II vestibular hair cells. There is some evidence that activation or deletion of transcriptional co-factors can alter hair cell identity – for example, activated b-catenin can produce vestibular-like hair cells in the chick basilar papilla [69], and mutation of the *Jxc1* transcription factor generates hair cells in the mouse cochlea with a vestibular morphology [92]. Moreover, since *Atoh1* is down-regulated in hair cells as they mature, it is not clear how many direct targets of *Atoh1* continue to be expressed in mature hair cells, nor how the expression of these genes is maintained.

Resolution of these questions requires identification of direct targets of *Atoh1* in hair cells. Unlike tissues such as the cerebellum, where *Atoh1*-expressing cells are present in large numbers and constitute a significant proportion of the total cell population, there are only a few thousand *Atoh1*-expressing hair cells in inner ear sensory organs, and they represent only a small minority of the total cell types. Thus, techniques such as chromatin immunoprecipitation and deep sequencing (ChIP-seq) are extremely difficult to apply to the inner ear. Nevertheless, it may be possible to exploit and apply recent data sets obtained from the cerebellum [91] to help identify *Atoh1* targets in the inner ear. For example, analysis of *Atoh1* targets in the cerebellum has identified a consensus Atoh1 binding motif based on an extended E box variant that is reminiscent of but not identical to that identified for *Drosophila atonal* (G/A C/A C A G/T C/A T G G/T C/T) [91]. This Atoh1 E-box associated motif, or AtEAM, is present close to the coding regions of over 65% of genes with sequences bound by Atoh1 in the cerebellum, and may be of use in predicting candidate Atoh1 targets in hair cells. However, as in *Drosophila*, it is possible that hair cell-specific targets may be regulated by a different E box variant. It is also clear that some of the processes regulated by *Atoh1* in cerebellar granule cells, such as proliferation and migration, do not occur in hair cells, and one might therefore expect significant differences in the repertoire of genes regulated by *Atoh1* in hair cells and granule neurons. Indeed, a recent comparison the ChIP-sequencing data from the cerebellum with gene expression data sets from cerebellar granule neurons, dorsal spinal interneurons and hair cells have identified only three genes – *Rab15*, *Selenoprotein M (Selm)* and *Atoh1* itself – that are candidates to be direct targets of Atoh1 in all three cell types [93].

Atoh1 is closely related to *Neurog1*, another bHLH factor that is necessary for the development of sensory neurons, including those generated by the inner ear [88,94]. Despite the similar sequence of both proteins, *Neurog1* is not able to fully substitute for *Atoh1*. Mice in which the coding sequence of *Atoh1* was replaced with *Neurog1* have a highly abnormal organ of Corti with very few hair cells [95]. The phenotype of these homozygous gene replacement mice is somewhat less severe than regular *Atoh1* null mice, suggesting that *Neurog1* is only able to activate a subset of *Atoh1* target genes. By analogy to the recently-discovered

AtEAM E-box binding variant in *Atoh1* target genes, it is likely that *Neurog1* has its own conserved DNA binding motif that is similar to, but distinct from, the AtEAM motif.

3.4: The function of Atoh1 in mechanosensitive Merkel cells

Although most attention has focused on the molecular function of *Atoh1* in hair cell development, *Atoh1* is also necessary for the development of Merkel cells, an epidermally-derived cell population believed to play a role in the discrimination of light touch [96,97]. Merkel cells associate with the neurites of SAI sensory afferents in the skin and bear large microvilli that interdigitate with epidermal cells. Merkel cells contain the actin-binding protein espin, (which is also seen in hair cell stereocilia), express a number of ion channels previously implicated in mechanotransduction, such as *PKD1*, *PKD2* and *TRPC1*, and show calcium influx in response to osmotic stress *in vitro* [98]. Together, these features suggest that Merkel cells may be intrinsically mechanosensitive [99], although this has yet to be directly demonstrated in the intact animal. Inactivation of *Atoh1*, either throughout the animal or specifically in the epidermis, causes a failure of Merkel cell development and an associated loss of SAI responses to light touch [8,9,100]. Little is known about the direct transcriptional targets of Atoh1 in Merkel cells, although it was recently shown that Merkel cells express *Rab15* and *Selm*, two *Atoh1* target genes also expressed in cerebellar granule neurons, spinal dP1/dI1 neurons and hair cells [93].

4: The role of Atoh1 in the regeneration of hair cells

Mammals are unable to regenerate their auditory hair cells after damage, and display only a very limited degree of regeneration in the vestibular system (reviewed in [101]). In contrast, non-mammalian vertebrates show robust hair cell regeneration after damage due to the proliferation and trans-differentiation of supporting cells. Non-mammalian vestibular sensory organs and the lateral line organs of teleosts also show a steady ongoing turnover of hair cells [102,103]. Accordingly, small numbers of *Atoh1*-expressing cells can be detected in these organs under normal conditions [104,105], and *Atoh1* is rapidly-re-activated in many

supporting cells during regeneration in non-mammalian vertebrates [102,104-106]. Regenerating hair cells quickly start to re-establish a normal proportion of hair cells and supporting cells through regulation of *Atoh1* by lateral inhibitory Notch signaling [105,107]. Inhibition of the Notch signaling pathway can further increase the proportion of cells expressing *Atoh1* in the chicken basilar papilla [106], and also in the adult mouse utricle [108], albeit to a much smaller degree.

The necessity and sufficiency of *Atoh1* for hair cell development, and the similarities in the regulation of *Atoh1* during hair cell development and regeneration has prompted much interest in using *Atoh1* as a potential target of gene therapy to promote hair cell regeneration in humans [109,110]. However, recent work has suggested that although *Atoh1* is sufficient to generate new hair cells, the ability of inner ear tissue to respond to *Atoh1* over-expression in this manner declines rapidly with age. We describe some of these experiments below and discuss possible reasons for the age-dependent loss of responsiveness to *Atoh1* activation

4.1: Is there an age-dependent limit on the ability of Atoh1 to induce hair cell differentiation in mammals?

As described above, *Atoh1* is one of the first genes to be up-regulated in supporting cells following hair cell loss in birds and fish. There are no reports of *Atoh1* being expressed in the mammalian cochlea after hair cell loss, although a small amount of *Atoh1* transcription has been seen in the mammalian vestibular system after damage, both in vivo and in vitro [108,111] – for example, an average of about 200 cells activate reporter gene expression from an *Atoh1* enhancer in the drug-damaged adult mouse utricle [108]. However, in both studies, only a very small fraction of *Atoh1*-expressing cells – typically less than 5% - go on to express Atoh1 protein or develop as hair cells [108,111]. Although this number can be increased somewhat by inhibiting Notch signaling [108], these results suggest that transcription from the *Atoh1* locus and translation of *Atoh1* mRNA are subject to significant impediments in the adult mammal.

In an attempt to overcome at least some of these limitations in Atoh1 expression in mammals, a number of studies have used adenoviral or transgenic expression of Atoh1 to generate new hair cells. Although a few

studies have shown that adenovirally-transduced *Atoh1* has the ability to restore hair cells in animals treated with ototoxic drugs [109,110,112], it is less clear whether this represents the generation of new hair cells or the repair of damaged, surviving hair cells, and further studies are needed to replicate and extend these findings. Recently, two studies used transgenic mice to activate *Atoh1* expression throughout the inner ear epithelium or specifically in sub-populations of supporting cells [55,113]. In both cases, expression of *Atoh1* in supporting cells or in non-sensory cochlear epithelium was able to induce new hair cells, some of which possessed stereociliary bundles and displayed voltage-dependent currents [55,113]. However, in both studies, the ability of *Atoh1* to induce new hair cell formation in all regions of the cochlea declined precipitously with age and was effectively abolished by two weeks of age, when hearing begins in mice [55,113]. This failure was also seen when Atoh1 was activated in supporting cells of adult mice in which hair cells had been killed with ototoxic drugs [113].

Why do supporting cells in the mature mammalian ear fail to respond to the activation of *Atoh1*? First, the Atoh1 protein itself may be post-translationally modified, for example by phosphorylation at its serine-rich C-terminal domain [59]. Second, it is known that inhibitory helix-loop-helix family members such as the Id proteins can block or attenuate bHLH gene activity by competing for E proteins, and that Id family members in the inner ear are known to antagonize Atoh1 function during development [114]. It is therefore possible that sustained expression of Id family members in the adult would block ectopically expressed Atoh1 activity. Third, it is possible that in addition to forming heterodimers with E proteins, such as E12 or E47, Atoh1 also requires other transcription factors or co-activators to regulate its targets. Such co-operating factors might be down-regulated in supporting cells with age, with the results that ectopically-expressed Atoh1 protein would not be able to activate its targets alone. Finally, it is possible that Atoh1 is active and functional when over-expressed mature mammalian supporting cells, but that its direct transcriptional targets have undergone epigenetic modification, rendering them unavailable for transcription. Some form of epigenetic reprogramming would therefore be required to allow supporting cells to transdifferentiate in response to *Atoh1* expression. This might involve changes in DNA methylation, or in the post-translational modification of histones to replace inhibitory epigenetic marks with

marks associated with active chromatin. Alternatively, Atoh1 could be used to reprogram supporting cells directly in concert with other, as-yet unidentified factors, by analogy to the reprogramming of fibroblasts into differentiated cells of the nervous system, muscle or pancreas. However, such approaches will first require the identification of more direct targets of Atoh1. In conclusion, it is clear that *Atoh1* has great potential as a therapeutic agent, but much more information is required about its regulators, cofactors, and targets in order to capitalize on this potential. The study of *atonal* family genes continues to be an exciting and important area of research.

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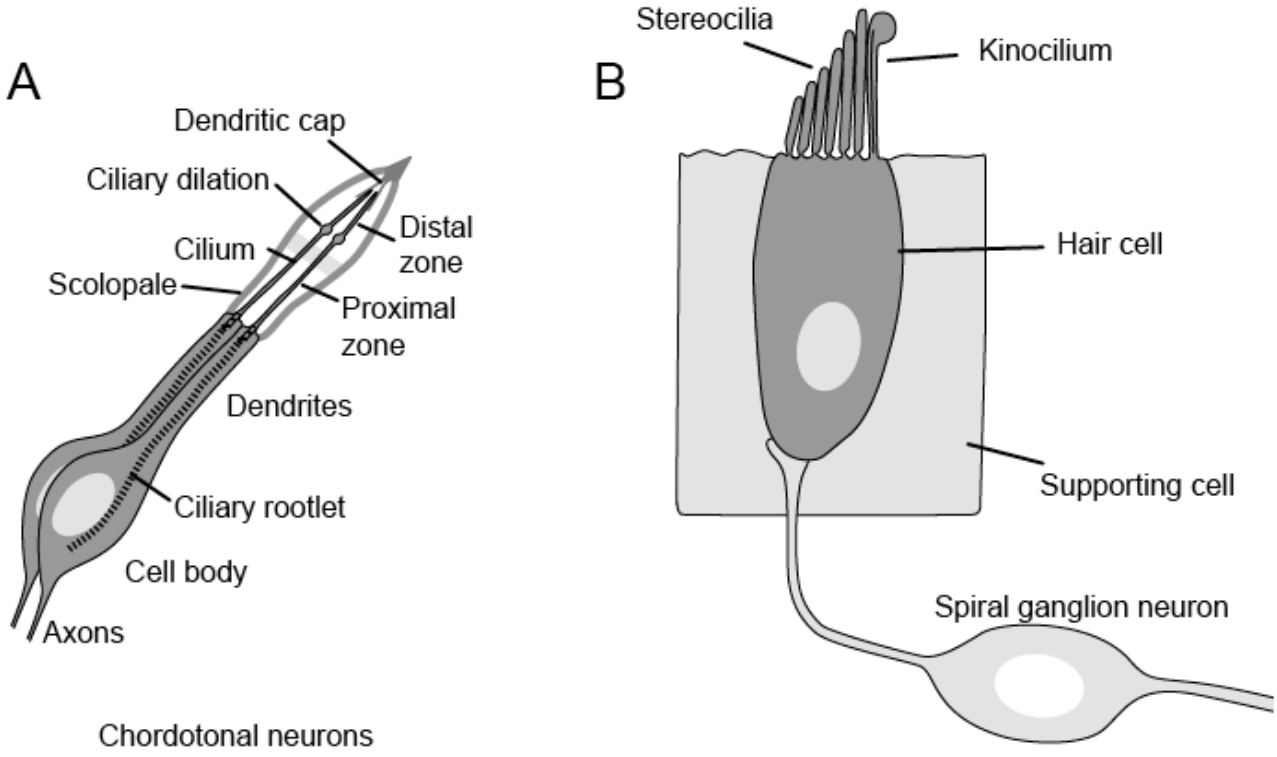

Figure 1: Mechanosensory cells that require *atonal* family genes. (A) The *Drosophila* Ch neuron. A Ch organ consists of 1-2 neurons and several support cells. One of the latter forms the characteristic scolopale 'basket' that houses the distal part of the Ch neuron dendrites. The monopolar dendrites terminate in a specialized mechanosensory cilium with functionally distinct zones. (B) The vertebrate hair cell. The mechanosensory apparatus consists of microvilli-based stereocilia. The cells also bear a true cilium (kinocilium). In cochlear hair cells the kinocilium may be required developmentally to organize the stereocilia although it later degenerates. In vestibular hair cells the kinocilium is retained, but its role in mechanosensation is not clear. Hair cells contact sensory neurons of the spiral ganglion. The sensory neurons require the bHLH factor, Neurog1, rather than Atoh1.

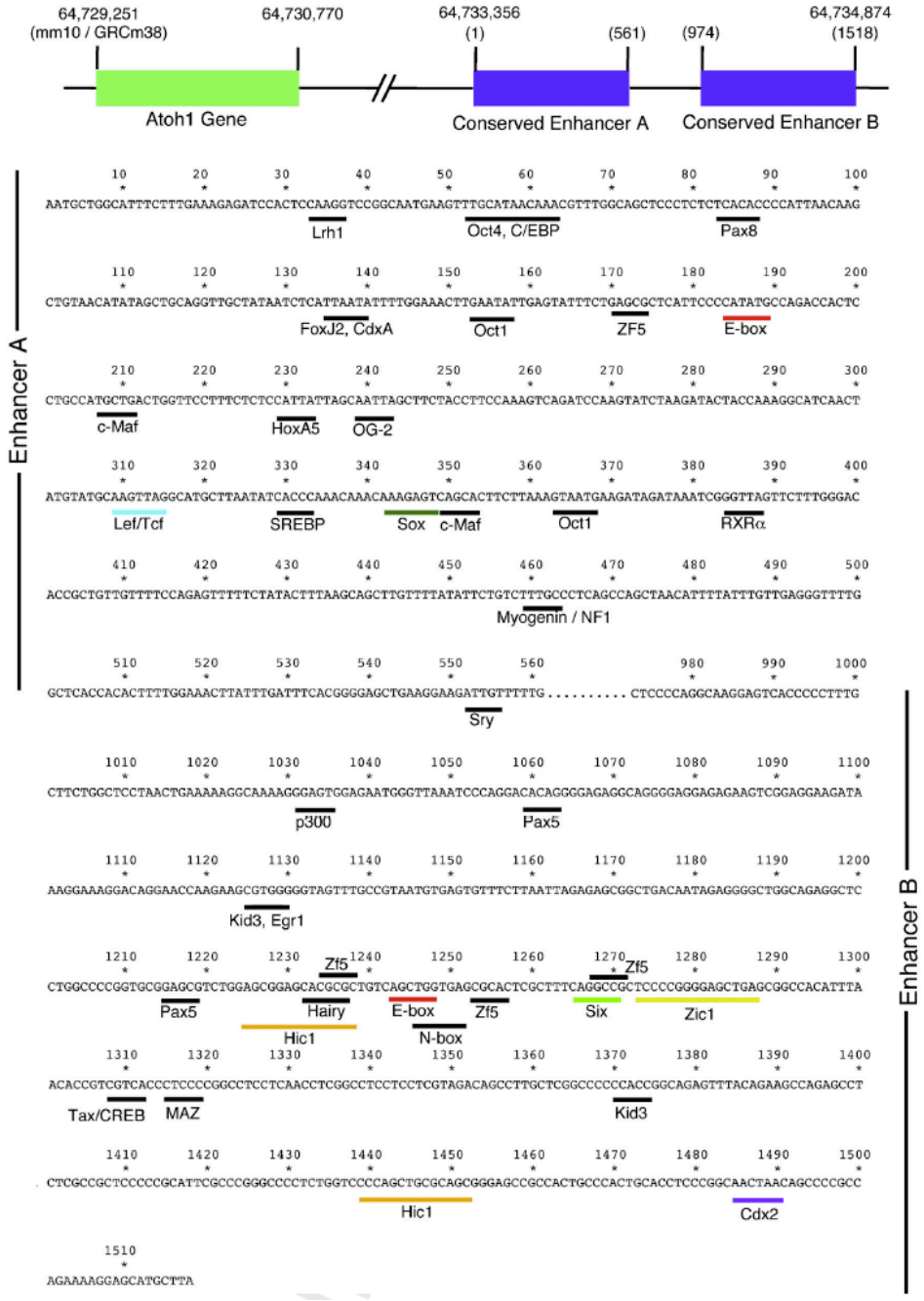

Figure 2: A diagram of the mouse Atoh1 locus, showing the position of its 3_ autoregulatory enhancer. Numbers above elements on the diagram refer to the position on mouse chromosome 6, according to the current build of the mouse genome (mm10/GRCm38). The Atoh1 autoregulatory enhancer consists of two conserved elements, A and B, with features of these enhancers numbered in parentheses relative to the start of enhancer A. Transcription factor binding sites that have been experimentally verified are shown in color on relevant regions of the enhancer sequence, with the remaining unverified but predicted sites shown in black.